As. J. Food Ag-Ind. **2011**, 4(06), 349-358

OPEN ACCESS

Asian Journal of Food and Agro-Industry

ISSN 1906-3040

Available online at www.ajofai.info

Research Article

Application of modified drop plate technique (MDPT) and logistic model to optimize non-selective substrates for *Salmonella typhi* resuscitation

Juthamas Khueankhancharoen and Aluck Thipayarat*

Department of Food Engineering, King Mongkut's University of Technology Thonburi, Bangkok, Thailand.

*Email: athipaya@yahoo.com

This paper was originally presented at the 12th ASEAN Food Conference, Bangkok, Thailand, 2011.

Abstract

The use of modified drop plate technique (MDPT) for aerobic cell enumeration together with sigmodial-type model simulation was proposed as an effective method to select suitable substrates (i.e., carbon and nitrogen sources) to maximize *Salmonella typhi* growth. This technique was able to accelerate the optimization of a medium recipe that allows a microorganism to grow at its best using a suitable enrichment medium. The modified drop plate utilized only 0.3 ml of agar substrate and 5 µl of inoculum. The incubation conditions were identical to the spread plate technique (37^{o}C); however, the detection time was significantly reduced from 18-24 h to only 8-10 h. The faster time to detect colonies was assisted by the use of a digital microscope and image analysis software. The comparison of the total plate count obtained from the modified drop plate (MDPT) and the spread plate technique (SPT) showed the equivalent number of final colony counts. This developed enumeration of forming colonies under the aerobic plate cultivation assisted by the use of a mathematical model (i.e. a logistic model) facilitated the counting method and also reduced the lengthy analytical time to optimize non-selective enrichment substrates for *Salmonella typhi* growth. Several key kinetics parameters, including the maximum specific growth rate (μ_{max}), were extracted and compared among different enrichment broths. The results indicated that TSB and BPW supported the better growth of *Salmonella* than LB and NB. This was indicated by the higher values of the maximum specific growth rate (μ_{max}) and achievable final cell concentration. It was surmised that the buffer system was able to stabilize the acidity, allowing the cells to grow at the most optimal condition.

Keywords: *Salmonella* spp., colony counts, micro-inoculation, logistic model, non-selective enrichment broth, Thailand.

Introduction

Salmonella is widely considered one of the most serious pathogenic contaminants present in products derived from poultry, eggs and meat. Occasionally this lethal pathogen can cause widespread disease outbreaks that have been documented in many countries all over the world [1, 2]. The US Centers for Disease Control and Prevention (CDC) recently reported *Salmonella* outbreaks were responsible for approximately 43,000 cases of food-transmitted salmonellosis (with a loss of 2.7 billion$ annually) [3]. It is conceivable that the lack of inexpensive and fast *Salmonella* test kits contributes to high death tolls [4]. The popular standard analytical method (e.g, ISO 6579, Bacteriological Analytical Manual, etc.) still requires a lengthy analytical time, usually a minimum of 4 days and several additional days for the confirmation of presumptive results [5, 6]. The general protocol entails numerous enrichment and isolation steps; none of which is considered absolute. In the actual industrial food safety application, the majority of the food samples analyzed return negative results. There are demands for disruptive measures in reducing analytical time and providing cost effective protocols. Also the development of such measures should extend to confirmation of potentially positive results [7].

The standard detection protocol of *Salmonella* requires three essential steps: pre-enrichment, selective enrichment and plating. The key factor to increase the chance to detect *Salmonella*, especially injured cells resulting from processing stresses such as heat, cold, or dehydration, is the development of effective non-selective enrichment. A variety of enrichment substrates have been recommended for resuscitation of the injured cells [8, 9, 10]. In particular, lactose broth and buffered peptone water are among a few recommendations suggested for the pre-enrichment of sub-lethally injured salmonellae from dried and frozen food products as well as frozen meat samples [10, 11]. In this study, MDPT was proposed for fast *S. typhi* enumeration and detection. The new protocol can also be used to optimize growth substrates and study the growth kinetics of *Salmonella* cultivation.

Materials and Methods

Microorganism and preparation of inoculum

S. typhi was obtained from the culture collection of Department of Microbiology, King Mongkut's University of Technology Thonburi, Thailand. Cultures were maintained by monthly transfer on tryptic soy agar (TSA, Difco) slants and stored at 4±1ºC. After two successive transfers of the stock culture on TSA at 37ºC for 24 h, the activated culture was inoculated into 100 ml of tryptic soy broth (TSB, Difco) and incubated at 37ºC for 16 h at a shaking speed of 200 rpm. The cell-free supernatant was recovered by centrifugation (8000xg, 4ºC, 10 min), then washed twice with 0.01 M phosphate buffered saline (PBS, pH 7.4). The washed cells, suspended and diluted in PBS to ca log 3 CFU/ml, were used as the inoculum for comparing growth in standard enrichment broths.

Bacterial cell enumeration and validation in micro-scale cultivation

S. typhi activated pure culture was recovered in 100 ml TSB contained in a baffled flask at 37ºC for 16 h. Cells were harvested, washed and suspended in PBS to ca log 9 CFU/ml. These initial washed cell suspensions were enumerated by both the conventional spread plate technique (SPT) and proposed MDPT on TSA. The cultivation volumes were reduced from the normal volume of 100 ml (SPT) to only 5 µl (MDPT) and the agar utilization per sample was around 0.3 ml on agar in the covers of 96-well plates. All the samples were kept in a hot-air incubator at 37±2ºC. Colony expansions and cell amplifications on the 96-well microplate covers were detected and captured using a reflected light microscope equipped with a CCD camera (Figure 1) during incubation over 24 h compared with the traditional spread plate method. The ability of MDPT and SPT to detect colony formation was also compared at different initial cell concentrations (0-8 log CFU/ml) using microscale technique.

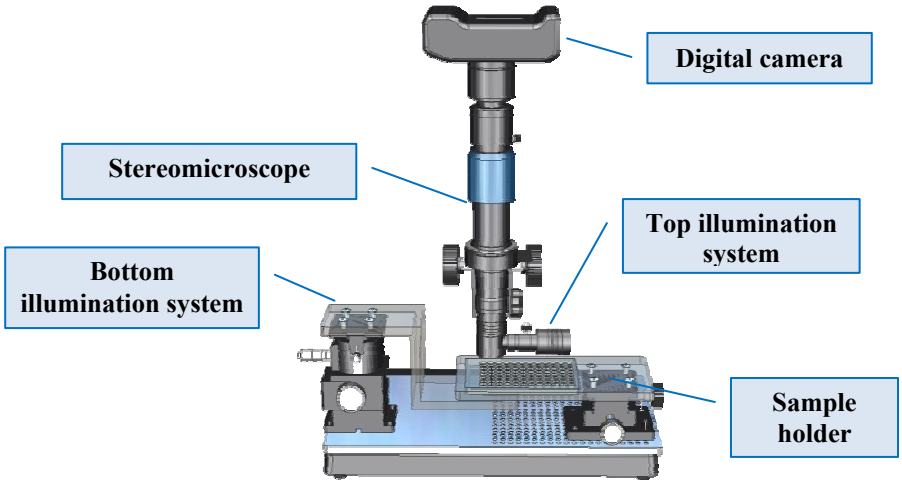


Figure 1. Image acquisition prototype of MDPT.

Comparison of standard enrichment broths

Various standard enrichment media for *Salmonella* (nutrient broth; NB, lactose broth; LB, buffered peptone water; BPW and tryptic soy broth; TSB), as specified by BAM, AOAC, FSIS, ISO etc., were evaluated to determine the effect of different media recipes on growth profiles of *S. typhi* at 37ºC. Growth curves were determined after a specific period of cultivation up to 24 h using 2 enumeration methods (the spread plate and modified drop plate technique).

Determining the growth kinetics using the mathematical model

The mathematical model (equation 1) has been successfully employed to predict the growth kinetics in batch cultivation including the maximum specific growth rate (μ_{max}), the inflection time (t_i) [12, 13].

$$x(t) = x_0 + \frac{x_m}{1+\exp} \quad (1)$$

where: x(t) is the cell concentration at any time (CFU/ml) of, t; x_o is the initial concentration (CFU/g); b is the maximum relative growth rate at t=N (h^{-1}); N is time at which the absolute growth rate is maximum (h). The parameter b can be used to define the specific growth rate of the growth; μ_{max} [14].

Statistical analysis

All data were analyzed at $p<0.05$ for significant values by ANOVA and Duncan's multiple range tests [15].

Results and Discussion

Bacterial cell enumeration and validation in microscale cultivation

Microscale cultivation was developed to substitute for the conventional cell count method (SPT). This technique was modified from the drop plate technique with the goal to fasten the colony count estimation and lower the associated analytical expense. The MDPT was compared to the common spread plate colony count. To verify the technique, *S. typhi* culture was prepared at approximately 9 log CFU/ml and applied to both MDPT and spread plate experiments for cell count. The SPT utilized the conventional petri dish format whereas the MDPT made use of a 96-well plate cover.

The results showed that when applying the MDPT, the time to detect *Salmonella* colonies was shortened to within 8 h (Figure 2). At this particular hour, no colonies were visually detected in the spread plate experiment. It took up to 16 h for the spread plate colonies to be visually detected and 18-24 h to achieve a steady cell count. Approximately, the detection time of the SPT lagged 8-14 hours behind that of the MDPT.

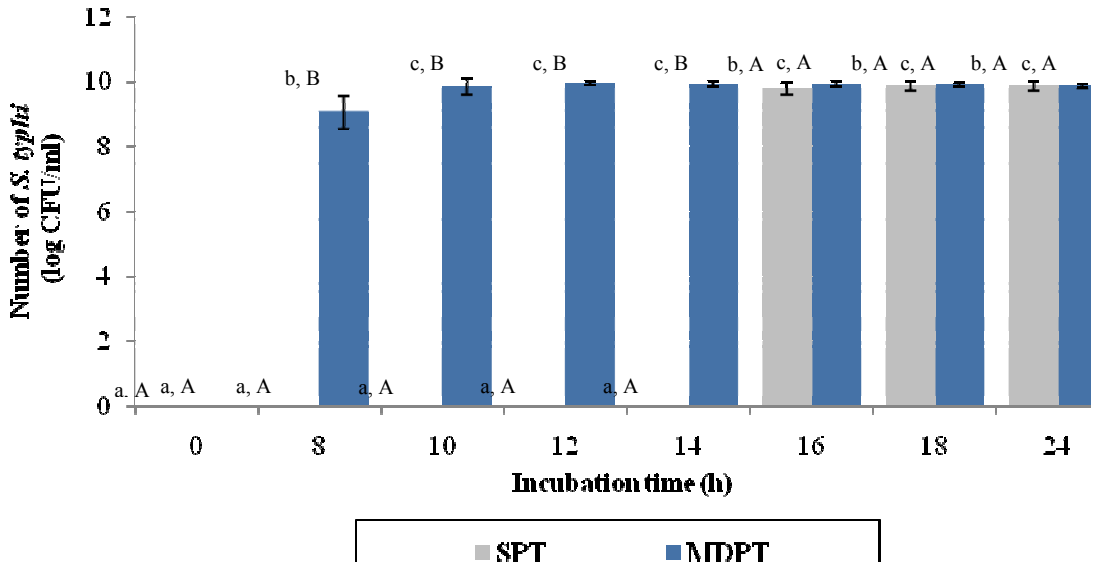


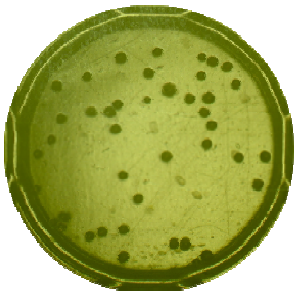
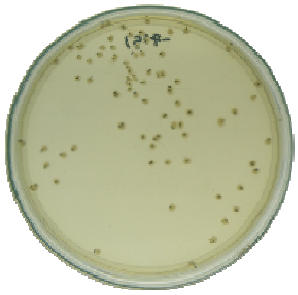
Figure 2. Number of *S. typhi* colonies in log CFU/ml enumerated by 2 methods (SPT and MDPT) during incubation time for 24 h.

Bar values with the same color but with different lowercase letters differ significantly. Bar values at any incubation time with different uppercase letters differ significantly (Duncan's multiple range test ($p< 0.05$)).

However, the colony count at the 8th hour from the MDPT was significantly less than the final approachable number (Figure 2). The MDPT required at least 10 hours for the colony count to be stabilized statistically (Table 1). Noting that this particular experiment was performed using active cells, the actual time for a steady cell count must be investigated case by case. The final cell count from the MDPT and the spread plate technique using the same stock culture was not statistically different at the 95% confident level.

The comparison of the SPT and MDPT detection on different concentrations of *Salmonella* was performed and the results were used to construct a standard curve as shown in Figure 3. The stock culture was prepared from 0 to 8 log CFU/ml. The MDPT returned practically identical cell counts as performed by the SPT. The slope of the standard curve was 0.9985 and the relative coefficient (r^2) was 0.9793. The variation of cell count values was very narrow except at the very low concentration. At the 2 log CFU/ml, the cell count variation was larger than at higher concentrations since the MDPT was performed in the proximity of its lower detection limit. At the 1 log CFU/ml, the MDPT exceeded its detection limit; hence, the method showed a zero cell count. The inoculum size of the MDPT was only 5 µl as opposed to 100 µl in the SPT. There were two orders of magnitude difference inherently associated with the inoculum size. This MDPT was previously applied to enumerate various strains of bacteria, for example *Listeria* spp. and *E. coli* [16-17].

Table 1. Comparison between the proposed MDPT under constructed light microscope and conventional SPT detected by human visualization.

Enumeration method	MDPT	SPT
		
Time to detect (h)	10-12	18-24
Number of *S. typhi (log CFU/ml)**	9.85 ± 0.25^{A}	9.77 ± 0.18^{A}

*Values in the same row with different letters differ significantly according to Duncan's multiple range test ($p < 0.05$).

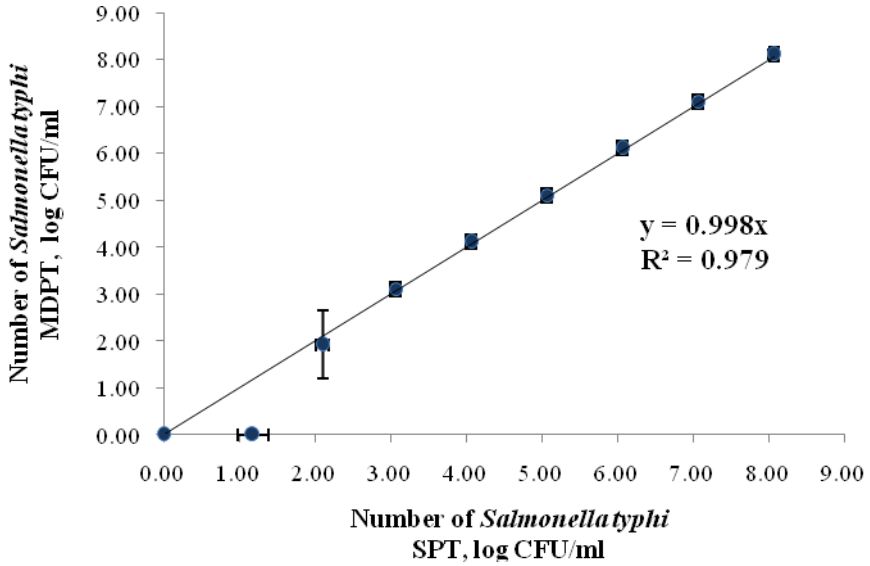


Figure 3. Comparison of a conventional SPT and MDPT method in *S. typhi* enumeration on TSA after incubation at 37ºC.

Validation of MDPT and SPT on pre-enrichment broths for* Salmonella *resuscitation

In Figure 4, the MDPT and SPT were applied and compared to capture the growth profiles of *Salmonella* enriched in different standard media, including NB, LB, BPW and TSB. The results confirmed the earlier finding that both MDPT and SPT generated growth profiles with a very high correlation. The cell count numbers at any given time were not significantly different ($p \geq 0.05$) in all media treatments.

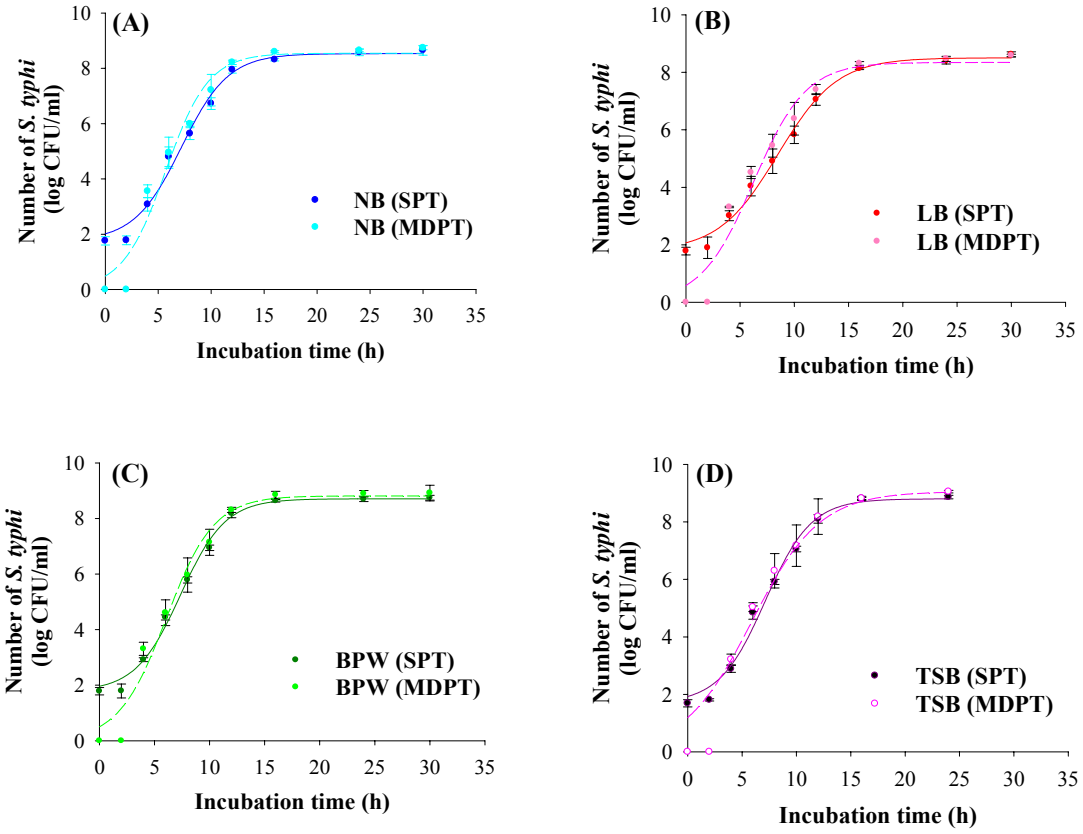


Figure 4. Growth of *S. typhi* in various standard enrichment media (A: NB; B: LB; C: BPW and D: TSB) incubated at 37ºC incubated for 24 h enumerated by 2 methods (SPT vs MDPT).

Generally, both protocols gave every small standard deviations; however, the exponential phase intrinsically possessed a higher variation of cell count due to the asynchronous nature of the stock culture. This large variation subsided as the cell growth reached its stationary phase. Only at the early stages of growth did the two techniques differ. MDPT returned no colony counts, whereas the SPT detected a few colonies in the order of 2 log magnitude. Again this happened because the detection limit of the MDPT was around 2 log CFU/ml as explained earlier. Nevertheless, the detection range of the rest of the growth profile was distant from this critical limit and suggesting that MDPT yields the same results as SPT.

***Study of non-selective growth kinetics for* Salmonella**

Applying the logistic model (eq.1) to simulate the growth profiles, this sigmoidal-type model fit well to the unique s-shape characteristics excluding the death phase (Figure 5). The growth kinetics of *Salmonella* on different traditional enrichment broths (i.e. NB, LB, BPW and TSB) were estimated, including the maximum cell density (x_{max}), the maximum specific growth rate (μ_{max}), and the inflection time (t_0). The log-scale increment from the initial cell loading (2 log CFU/ml) to 5 log CFU/ml of the final cells was achieved within 10 h. The rapid growth during the pre-enrichment step was desirable; it promoted the target cell survival over the strong toxicity during the selective enrichment [18]. Preferably, the pre-enrichment should result in the proliferation of cell population at least 5 log CFU/ml prior to the selective enrichment process.

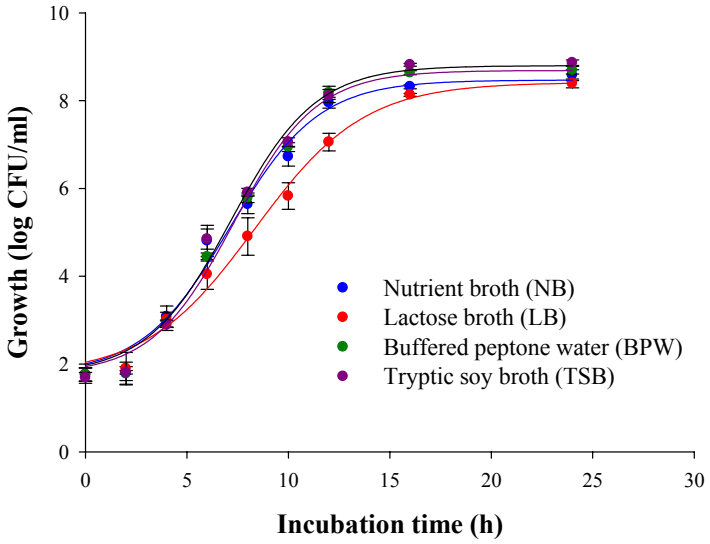


Figure 5. Growth of *S. typhi* in various standard enrichment media (NB, LB, BPW and TSB) incubated at 37ºC for up to 24 h.

However, most standard protocols (e.g. BAM, ISO, etc.) prolong the incubation time to 16-24 h or overnight to ensure ample cell density [19]. A suitable cell density for any subsequent selective agar plating is a subtle factor in determining an appropriate enrichment period. The concern over the most effective choice of non-selective broth for *Salmonella* resuscitation was less critical [20]. Figure 5 shows all standard media returned similar growth profiles. Many studies comparing several nonselective media also showed a comparable proliferability [21]. The more relevant problem is the concern of the microbial population presented in real food products. After sub-lethal processing, that population most likely includes not only normal cells but also injured microorganisms (stressed, sub-lethally or reversibly injured) [22, 23]. The resuscitation and detectability of these injured cells is more pertinent to optimize the requirement of the non-selective enrichment.

Figure 6 summarizes the key kinetic parameters extracted from the logistic function. The BPW and TSB growth profiles produced no statistical differences in x_{max}, μ_{max} and t_0 meanwhile the LB treatment significantly revealed less desirable values in all parameter comparisons to the other media. Growth kinetics in NB were more desirable than those in LB though TSB yielded the highest final cell numbers. pH fluctuations due to metabolites produced by microorganisms are known to occur [24, 25]. Moreover, the effect of LB is the alteration of acidity because *S. typhi* belongs to one of the lactose-positive *Salmonella* strains [26]. This microorganism can utilize the lactose presented in LB as an energy source and produce organic acids lowering pH in the broth. Hoffman *et al.* [27] demonstrated the potential resuscitation of *Salmonella* in lactose-containing media was significantly less when the final pH reached 4.4. Without lactose, the final pH decreased to only 5.7 and *Salmonella* grew to a higher cell density. In addition to providing conditions for the resuscitation and growth of cells prior to selective enrichment, BPW buffers the pH of the growth system against pH changes brought about by the growth and metabolism of micro-organisms during enrichment and those imposed by the food sample.

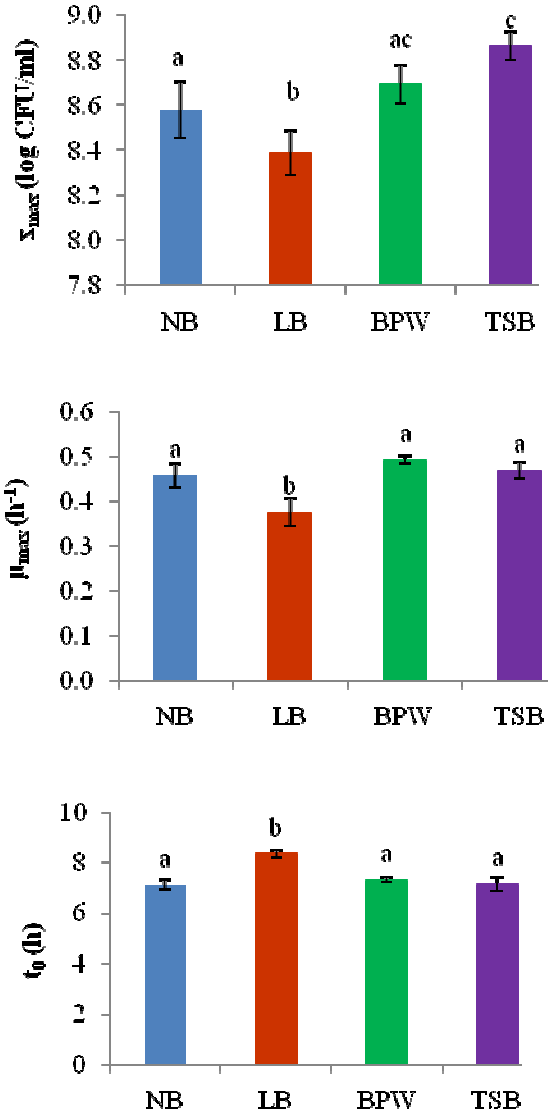


Figure 6. The kinetic parameters (A: X_{max}; B: μ_{max} and C: t_0) of *S. typhi* in the presence of various standard enrichment media (NB, LB, BPW and TSB) incubated at 37°C for 24 h.

Conclusion

The study of different pre-enrichment media for *Salmonella* pointed to the replaceablity of any standard non-selective substrates. Among the selected generic media used, LB should be the least preferable medium due to the promotion of acid production from lactose. This intensive study was facilitated by the development and application of the MDPT. The micro-scale cultivation allowed the efficient use of media and supplies. Also, the detection time of visualized colonies was significantly reduced using digital microscopic detection. The MDPT was successfully validated against the SPT with a high repeatability and good accuracy.

Acknowledgements

The graduate scholarship support from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0145/2551) to Juthamas Khueankhancharoen and Dr. Aluck Thipayarat is acknowledged. The authors are grateful to have strong industrial partnership

with Buono (Thailand) Co.,Ltd and would like to express sincere appreciation to Mrs. Achara Jiamthaworn and Mr. Chettha Supparasuwat for their kind collaboration through the course of our research.

References

1. Favrin, S.J., Jassim, S.A. and Griffiths, M.W. (2003). Application of a novel immunomagnetic separation-bacteriophage assay for the detection of *Salmonella enteritidis* and *Escherichia coli* 0157:H7 in food. ***International Journal of Food Microbiology.*** 85: 63-71.

2. Borowsky, L.M., Schmidt, V. and Cardoso, M. (2007). Estimation of most probable number of *Salmonella* in mince pork samples. ***Brazilian Journal of Microbiology***. 38: 544-6.

3. CBCNEWS. 2009. Recall in U.S. *Salmonella* outbreak expands to more than 125 products. Available from: http://www.cbc.ca/news/. Accessed Nov 11.

4. Patel, P.D. and Williams, D.W. 1994. Evaluation of commercial kits and instruments for the detection of foodborne bacterial pathogens and toxins. In: Patel PD, (ed.). Rapid analysis techniques in food microbiology. Chapman & Hall. Glasgow, Scotland. 294p.

5. USFDA (United State Food and Drug Administration). (2007). Bacteriological Analytical Manual (BAM): Chapter 5 *Salmonella*. Retrieved from: http://www.cbc.ca/news/ (Accessed September 22, 2010).

6. Vasavada, P.C. (1997). Advances in pathogen detection. ***Food Testing and Analysis***. 3: 18–20, 22–3, 47.

7. Oggel, J.J., Nundy, D.C., Ann Zebchuk, P. and Shaw, S.J. (1995). Reliability of the modified semi-solid Rappaport–Vassiliadis agar and the modified 1–2 test system for detection of *Salmonella* in poultry feeds. ***Journal of Food Protection***. 58: 98–101.

8. Allen, L.A., Pasley, S.M., and Pierce, M.S.F. (1952). Conditions affecting the growth of Bacterium coli on bile salts media. Enumeration of this organism in polluted water. ***Journal of General Microbiology***. 7: 257-67.

9. Bissonnette, G.K., Jezeski, J.J., McFeters, G.A. and Stuart, D.G. (1975). Influence of environmental stress on enumeration of indicator bacteria from natural waters. ***Applied Microbiology***. 29: 186-94.

10. Edel, W. and Kampelmacher, E.H. (1973). Comparative studies on the isolation of "sub-lethally injured" salmonellae in nine European laboratories. ***Bulletin W.H.O.*** 48: 167-74.

11. North, W.R. (1961). Lactose pre-enrichment method for isolation of *Salmonella* from dried egg albumen: Its use in a survey of commercially produced albumen. ***Applied Microbiology***. 9: 188-95.

12. Brewster, J.D. (2003). A simple micro-growth assay for enumerating bacteria. ***Journal of Microbiological Methods***. 53: 77 – 86.

13. Juneja, V.K., Melendres, M.V., Huang, L., Subbiah, J., Thippareddi, H. (2009). Mathematical modeling of growth of Salmonella in raw ground beef under isothermal conditions from 10 to 45^{o}C. ***International Journal of Food Microbiology***. 131: 106–11.

14. McMeekin, T.A., Chandler, R.E., Doe, P.E., Garland, C.D., Olley, J., Putro. S. and Ratkowsky, D.A. (1987). Model for combined effect of temperature and salt concentration/ water activity on the growth rate of *Staphylococcus xylosus*. ***Journal of Applied Microbiology***. 62: 543-50.

15. Statistical Analysis System. (1983). SAS Introductory Guide. SAS Institute, Cary, NC, 93p.

16. Khueankhancharoen, J., Supanivatin, P., Saeung, W., Boonyaprapasorn, A. and Thipayarat, A. (2010). Evaluation of practical analyses of coliforms and *Escherichia coli* detection and enumeration for industrial food samples. Proceedings of International Conference for a Sustainable Greater Mekong Subregion; Aug 26-7, Bangkok, Thailand, 846 p.

17. Naraipitak, S. (2009). Optimization and growth kinetics of *Listeria* spp. in micro-cultivation environment for rapid detection (A Special Research Project). Bangkok: King Mongkut's University of Technology Thonburi. 74 p. Available from King Mongkut's University of Technology Thonburi.

18. Chen, H., Fraser, A.D.E. and Yamazaki, H. (1993). Evaluation of toxicity of *Salmonella* selective media for shortening the enrichment period. ***International Journal of Food Microbiology***. 18: 151-9.

19. D'Aoust, J.Y., Sewel, A.M. and Warburton, D.W. (1992). A comparison of standard cultural methods for the detection of foodborne *Salmonella*. ***Journal of Food Microbiology***. 16: 41-50.

20. Doyle, M.P. (Ed.). 1989. *Salmonella*. In: Foodborne Bacterial Pathogens. Marcel Decker Inc., New York. 816p.

21. D'Aoust, J.Y. and Maishment, C. (1979). Pre-enrichment conditions for effective recovery of *Salmonella* in foods. ***Journal of Food Protection***. 44: 369-74.

22. Ray, B. (1979). Methods to detect stressed microorganisms. ***Journal of Food Protection***. 42: 346-55.

23. Wu, V.C.H. (2008). A review of microbial injury and recovery methods in food. ***Food Microbiology***. 25: 735-44.

24. Baylis, C.L., MacPhee, S. and Betts, R.P. (2000). Comparison of two commercial preparations of buffered peptone water for the recovery and growth of *Salmonella* bacteria from foods. ***Journal of Applied Microbiology***. 89: 501-10.

25. Sadovski, A.Y. (1977). Technical note: Acid sensitivity of freeze injured salmonellae in relation to their isolation from frozen vegetables by pre-enrichment procedure. ***International Journal of Food Science and Technology***. 12: 85-91.

26. Kunz, L.J. and Ewing, W.H. (1965). Laboratory infection with a lactose-fermenting strain of *Salmonella typhi*. ***Journal of Bacteriology***. 89: 1629.

27. Hoffman, C.A., Wagner, D.E. and Tatini, S. (1997). Comparative study of recovery of *Salmonella javiana* from mozzarella cheese by two official analytical procedures. ***Journal of Food Protection***. 60: 1493-6.